

# EXHIBIT A


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## United States Adopted Names

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The purpose of the United States Adopted Names (USAN) Council is to serve the health professions in the United States by selecting simple, informative, and unique nonproprietary names for drugs by establishing logical nomenclature classifications based on pharmacological and/or chemical relationships.

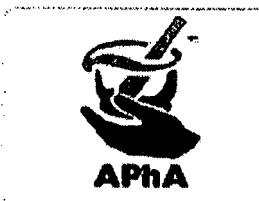
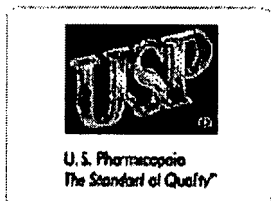
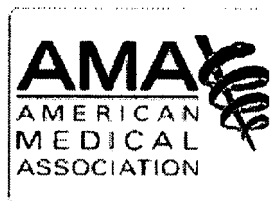
The USAN Council (tri-sponsored by the American Medical Association (AMA), the [United States Pharmacopeial Convention](#) (USP), and

the American Pharmacists Association (APhA), aims for global standardization and unification of drug nomenclature and related rules to ensure that drug information is communicated accurately and unambiguously, working closely with the [International Nonproprietary Name \(INN\) Programme](#) of the World Health Organization (WHO), and various national nomenclature groups.

### Newly approved USAN

The following approved USAN have been released for publication:  
 (PDFs require [Adobe® Reader®](#))

[iscotrizinol](#) (17KB)



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### USAN application process

Considerations and requirements before, during and after a USAN is recommended and adopted.

### Staff contacts

Obtain answers to your general questions to expedite the USAN process.

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**Approved stems**

List of common stems for which chemical and/or pharmacological parameters have been established.

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**Published names**

A listing of adopted USAN.

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**Names under consideration**

A listing of the nonproprietary names for the drugs described that are under consideration by the USAN Council.

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**Naming biologics**

Guidelines for assigning USAN for biological products such as insulins, interferons, interleukins, growth hormones, colony-stimulating factors, cytokines, monoclonal antibodies, and coagulation factors.

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**Contact lenses**

This section describes how to apply for a USAN for a contact lens material.

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**Gene therapy naming scheme**

Gene therapy naming scheme to assign names to non-cellular products produced by insertion of genetic material (transgene) into a vector (virus or plasmid) and where altered genetic material is administered to patients as a biologic drug. This scheme distinguishes a gene therapy drug and conveys safety information to the user physician.

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**Cell therapy naming scheme**

The nomenclature scheme for cell therapy products, described in this report, was developed by the USAN working group for cell therapies. The naming scheme would apply to all cell therapy products, with the exception of minimally manipulated hematopoietic elements, combination products and prophylactic vaccines. Since most cell therapy products are manipulated or modified in some way, the manipulation or modification would be considered as part of the product and would hence be part of the name.

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**Organic radicals, counterions and solvent molecules used in coining two-word names**

Listing which includes terms for common and uncommon radicals and anions compiled by USAN for the purpose of providing assistance and guidance in selecting appropriate two-word names when working with salts and esters.

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**Geometric isomerism and chirality: the USAN perspective**

This document explores the "USAN experience" with substances that have a chiral center and explains nomenclature rules for enantiomers and stereoisomers. We analyzed records of USAN adoptions between 1999 and 2004 to see how many chiral compounds were undergoing clinical development.

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The USAN Council has been involved in coining names for various biological products: the insulins, interferons, interleukins, growth hormones, colony-stimulating factors, cytokines, monoclonal antibodies, and coagulation factors. With increasing development of highly purified biological extracts and recombinant materials, the Council has had an increasingly greater role in assigning names and developing nomenclature rules for these agents.

**Interferons****Interleukins**

nakin, onakin, benakin, leukin, trakin, interleukin, penkin, exakin, eptakin, octakin, nonakin, decakin, elvekin, dodekin

**Somatotropins****Erythropoietins****Monoclonal antibodies**

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**Monoclonal antibodies**
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The following guidelines have been developed for monoclonal antibodies:

- The suffix *-mab* is used for monoclonal antibodies and fragments.
- Identification of the animal source of the product is an important safety factor based on the number of products that may cause source-specific antibodies to develop in patients.

The following letters were approved as product source identifiers:

u = human	e = hamster
o = mouse	i = primate
a = rat	xi = chimera
zu = humanized	

These identifiers are used as infixes preceding the *-mab* suffix stem, eg:

- <u>u</u> mab (human)	- <u>xi</u> mab (chimera)
- <u>o</u> mab (mouse)	- <u>zu</u> mab (humanized)

- The general disease state subclass must be incorporated into the name by use of a code syllable. The following disease state subclasses were approved based on products currently before the Council. Additional subclasses will be added as necessary.

**Disease or Target Class:**

Viral	- <i>vir</i> -
Bacterial	- <i>bac</i> -
Immune	- <i>lim</i> -
Infectious Lesions	- <i>les</i> -
Cardiovascular	- <i>cir</i> -

**Tumors**

colon	- <i>col</i> -
melanoma	- <i>mel</i> -
mammary	- <i>mar</i> -
testis	- <i>got</i> -
ovary	- <i>gov</i> -
prostate	- <i>pr(o)</i> -
miscellaneous	- <i>tum</i> -

- In order to create a unique name, a distinct, compatible syllable should be selected as the starting prefix.
- Sequence of stems: The order for combining the key elements is as follows: Infix representing the target disease state, the source of the product, and the monoclonal root *-mab* used as a suffix (eg, *biciromab*,

*satumomab, nebacumab, sevirumab, tuvirumab*). When combining a target or disease infix stem with the source stem for chimeric monoclonal antibody, the last consonant of the target/disease syllable is dropped, eg:

TARGET	SOURCE	-MAB STEM	USAN
-cir-	-xi	-mab	<i>abciximab</i>
-lim-	-zu	-mab	<i>daclizumab</i>

These modifications were deemed necessary to facilitate pronunciation of the resultant designation.

- If the product is radiolabeled or conjugated to another chemical such as a toxin, identification of this conjugate is accomplished by use of a separate, second word or other acceptable chemical designation. For monoclonals conjugated to a toxin, the "-tox" stem must be included as part of the name selected for the toxin (eg, zolimomab *aritox*, the designation *aritox* was selected to identify ricin A-chain). For radiolabeled products, the word order is: name of the isotope, element symbol, isotope number, and name of the monoclonal antibody: eg,

technetium Tc 99m *biciromab*  
indium In 111 *altumomab pentetate*

- A separate, distinct name must be assigned to any linker/chelator used to conjugate the monoclonal antibody to a toxin, isotope, or for pegylated monoclonal antibodies, eg,

*telimomab aritox*  
indium In 111 *satumomab pentetide*  
*enlimomab pegol*

For the USAN Council to initiate the selection of a nonproprietary name for a monoclonal antibody or fragment, the nomenclature application must provide the following relevant information:

1. The immunoglobulin class and subclass and the type of associated light chain.
2. Identity of the fragment of the immunoglobulin used (if applicable).
3. Species source from which the coding region for the immunoglobulin originated and specific, complete origin of all parts of chimeric, humanized, or semisynthetic immunoglobulins.
4. The antigen specificity of the immunoglobulin, including its source.
5. The clone designation (specify if vector or vector-cell combination).
6. For conjugated monoclonal antibodies, the identity of any linkers, chelators, toxins, and/or isotopes present in the product.
7. Identity of other modifications to the antibody, eg, reduction of disulfide bonds, glycosylation or deglycosylation, amino acid modification, or substitution.

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# EXHIBIT B

## RESEARCH/

# BY-PASSING IMMUNIZATION: BUILDING HIGH AFFINITY HUMAN ANTIBODIES BY CHAIN SHUFFLING

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Jacqueline M. Bye<sup>1,2</sup> and Greg Winter<sup>1,3,\*</sup>

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Diverse antibody libraries can be displayed on the surface of filamentous bacteriophage, and selected by panning of the phage with antigen. This allows human antibodies to be made directly *in vitro* without prior immunization, thus mimicking the primary immune response<sup>1</sup>. Here we have improved the affinity of one such "primary" antibody by sequentially replacing the heavy and light chain variable (V) region genes with repertoires of V-genes (chain shuffling)<sup>2</sup> obtained from unimmunized donors. For a human phage antibody for the hapten 2-phenyloxazol-5-one (phOx) ( $K_d = 3.2 \times 10^{-7}$  M), we shuffled the light chains and isolated an antibody with a 20 fold improved affinity. By shuffling the first two hypervariable loops of the heavy chain, we isolated an antibody with a further 15-fold improved affinity. The reshuffled antibody differed in five of the six hypervariable loops from the original antibody and the affinity for phOx ( $K_d = 1.1 \times 10^{-9}$  M) was comparable to that of mouse hybridomas from the tertiary immune response. Reshuffling offers an alternative to random point mutation for affinity maturation of human antibodies *in vitro*.

**F**or serotherapy, monoclonal antibodies would ideally be of human origin, but human hybridomas are difficult to make and require human immunization (see ref. 3 for review). New technologies have prompted new solutions. For example, gene technology has prompted the 'humanizing' of rodent antibodies by transplanting their hypervariable loops into a human antibody<sup>4-8</sup>, leading to clinical application<sup>9</sup>. The use of the polymerase chain reaction<sup>10</sup> (PCR), to clone and express antibody V-genes<sup>11,12</sup> and phage display technology<sup>13,14</sup> to select antibody genes encoding fragments with binding activities<sup>15</sup> has resulted in the isolation of antibody fragments from repertoires of PCR amplified V-genes using immunized mice or

humans<sup>2,16</sup> thus by-passing conventional hybridoma technology.

Recently, we reported the isolation of human antibody fragments directed against both small (hapten) and large (protein) antigens from the same single chain Fv (scFv)<sup>17,18</sup> library ( $3 \times 10^7$  members) made from the V-genes of unimmunized healthy blood donors and displayed on the surface of bacteriophage<sup>1</sup>. The process bypasses immunization by mimicking immune selection. Indeed, the antibody fragments were highly specific and had affinities typical of a primary immune response ( $K_d = 1 - 5 \times 10^{-7}$  M). The technology appears to have the potential to make human antibodies entirely *in vitro*, but for most practical applications the antibodies need higher affinities typical of later immune responses.

Affinity maturation can be mimicked *in vitro* by making point mutations in the V-genes, for example by using an error-prone polymerase, and selecting mutants for improved affinity<sup>19</sup>. Alternatively, new combinations of antibody heavy and light chains can be made by recombining a single heavy or light chain with a library of partner chains (chain shuffling). Chain shuffling has been used to make new combinations of heavy and light chains with hapten binding activities from the V-genes of immunized animals but affinities of the shuffled antibodies were not measured<sup>20</sup>. An attempt to derive hapten binding antibodies by reshuffling the V-genes from an immunized source with those from a naive source failed, prompting the authors to assert that "redesign of antibodies through recombination of a somatically mutated chain with a naive partner may be a difficult process"<sup>20</sup>.

For this work, we started with the human antibody ( $\alpha$ phOx-15) directed against the hapten 2-phenyloxazol-5-one (phOx) that had been isolated from a phage display library made from unimmunized human donors<sup>1</sup>. Both heavy and light chains of  $\alpha$ -phOx-15 are somatically mutated. Using repertoires of heavy and light chain V-genes from unimmunized donors, we reshuffled the heavy chain with the repertoire of light chains, and vice-versa to make shuffled somatically mutated antibodies with higher affinities.

## RESULTS

**Light chain shuffling.** A scFv fragment ( $\alpha$ phOx-15) directed against the hapten phOx was isolated from a phage antibody library constructed from the heavy (VH) and light (Vk and VL) chain genes from the peripheral blood lymphocytes of unimmunized human donors<sup>1</sup>. The VH gene of  $\alpha$ phOx-15 was assembled with a repertoire of Vk and VL genes from the same unimmunized donors to make shuffled scFv genes<sup>2</sup>, and cloned into the phagemid vector pHEN1 (ref. 21) for display as a fusion with gene 3 coat protein<sup>15</sup>. After transformation, the pha-



Light Chain	Relative Affinity	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
$\alpha$ phOx-15	1.0	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQHLPGTAPNLLIY	DNNGQPS	GIPDRFSGSKSGISATLGITGLQTGDEADYYC	GTMDGRILTAAY	PGGGTKVTVL
JM1A (germline)		-----	-----	---Q---K---	-----	-----	---SS-S-G	-----
$\alpha$ phOx2	28.6	-----	-----	---VO---K---	---F---V---	---P---	---A---S-RE---	-----
$\alpha$ phOx2	10.0	-----	-----	---Q---K---	---N---	-----	---SS-SEG---	-----
$\alpha$ phOx27	7.4	-----	---R-G-TL---	---QV---R---	-----	-----	---SN-R-G---	-----
$\alpha$ phOx5	6.0	-----	-----	---Q---K---	-----	-----	---SS-S-G---	-----
$\alpha$ phOx8	5.0	-----	---R---	---Q---K---	---D---	-----	---SS-S-G---	-----
$\alpha$ phOx4	2.0	-----	-----	---Q---R---	-----	-----	---SS-S-V---	-----
$\alpha$ phOx6	1.5	-----G---R---	---R---	---Q---R---	-----	-----	---SS-S-V---	-----
Heavy Chain	Affinity (nM)	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
$\alpha$ phOx15	320	QVQLVQSGAEVKKPGASVKVSCKASGYTF	SYGIS	WVRQAPGQGLEWNG	WISAYNGMKYAKLQG	RVTMTDTSTSTAYNEIRLSRSDTAVYCYVR	LIPKRTATLHYIIDV	WGKGLTVVSS
VH380.6 germline		-----	-----	-----	-----	-----	-----	-----
$\alpha$ phOx31E	1	-----G-----	N-T	-----	-----YKS-NF---	-----F-----	-----	-----
$\alpha$ phOx312D	6	-----G-----	S-R	-----	---S-G-Q---FR---	-----R-----	-----	-----
$\alpha$ phOx48A	10	-----D-R-----	N-T	-----	-----S-I---	---T---K---L-VTN---	-----	-----
$\alpha$ phOx412D	15	-----	R-T	-----	---N-F---	---A---	-----	-----
$\alpha$ phOx34H	26	-----A-----	R-T	-----	---SA---	---N---	-----	-----

**FIGURE 1** Sequences and affinities of the light and heavy chains of phOx binders from the shuffled libraries. The sequences of the light chains are compared to  $\alpha$ phOx-15 and the most homologous VL germline gene, JM1A (ref. 1). The sequences of the heavy chains are compared to  $\alpha$ phOx-15 and the most homologous germline gene VH380.6 (ref. 1). Relative affinities were determined by inhibition ELISA and are expressed as

$I_{50}$  mutant/ $I_{50}$   $\alpha$ phOx15. Affinities were determined by fluorescence quench titration. All antibodies bound phOx specifically (did not bind BSA in an ELISA and binding to phOx-BSA coated microtitre plates could be inhibited by soluble phOx-GABA). \*Location of the cloning site for the heavy chain repertoire.

gemid library ( $2 \times 10^6$  clones) appeared diverse by BstNI fingerprinting<sup>2</sup>, was rescued with helper phage<sup>1</sup> and subjected to panning on phOx-BSA coated tubes<sup>1</sup>. For expression of soluble scFv, the phage eluted from the tubes were used to infect a non-suppressor strain of bacteria<sup>21</sup> (for details see Experimental Protocol).

To identify clones with improved affinities, the binding of soluble scFvs to phOx-BSA were compared by ELISA. After a single round of panning, soluble scFv from 59/192 clones bound to phOx-BSA with a stronger signal than  $\alpha$ phOx-15 scFv whereas before panning, none of 192 clones gave a stronger signal. Six of these clones, and a further 4 clones from a second round of panning, were sequenced. Six unique VL light chains were found, all from the same VL1-gene family and probably the same germ-line gene as the  $\alpha$ phOx-15 light chain (Fig. 1). The human VL chains were mutated at a range of sites, diverging by 0 to 9 amino acid residues from the putative VL germ-line gene (VJ1M1A). The clustering of residue changes, particularly in CDR3, indicates that the mutant light chains were derived directly from the germ-line VL-gene rather than the  $\alpha$ phOx-15 light chain (Fig. 1).

The scFv fragments from eight different clones were ranked by competition for binding to phOx-BSA with soluble phOx hapten<sup>22</sup>, and the "relative affinities" were

found to be up to 27 fold higher than  $\alpha$ phOx-15 (Fig. 1). The affinities of  $\alpha$ phOx-15 and  $\alpha$ phOx2 (the clone with the highest relative affinity) were also measured directly by fluorescence quench titration. The affinity of  $\alpha$ phOx2 was found to be  $1.5 \times 10^{-8}$  M (20 fold higher than  $\alpha$ phOx-15) (Table 1). The kinetics of binding (off-rates) of purified  $\alpha$ phOx-15 and  $\alpha$ phOx2 scFvs to phOx modified BSA were determined by real-time biospecific interaction analysis based on surface plasmon resonance (SPR, Pharmacia BIAcore)<sup>23,24</sup>. The off-rate was much slower for  $\alpha$ phOx2 but calculated on-rates ( $k_{on}/K_d$ ) were similar (Table 1). Thus the improved affinity of  $\alpha$ phOx2 is due to its slower off-rate.

**Heavy chain shuffling.** The reshuffled heavy chain library was prepared as described in the Experimental Protocol. Briefly, a repertoire of VH genes (VH1 family) was amplified by PCR from the IgG and IgM mRNAs of unimmunized donors using primers based in the first and third framework regions. The VH repertoire which encodes the first two hypervariable loops and three framework regions, was cloned into a vector encoding the third hypervariable loop and the light chain of  $\alpha$ -phOx2. The resulting library ( $2 \times 10^6$  clones) was panned on phOx and soluble scFv screened by ELISA for binding after each round of selection.

**TABLE 1** Affinities and kinetics of binding to phOx of original isolate ( $\alpha$ phOx15) and chain shuffled mutants.

Clone	Residue changes (from $\alpha$ phOx-15)	$K_d$ *(M)	$k_{on}$ †(s <sup>-1</sup> )	$k_{off}$ §(M <sup>-1</sup> s <sup>-1</sup> )
Original Isolate $\alpha$ phOx15	0	$3.2 \pm 0.1 \times 10^{-7}$	$4.3 \pm 0.6 \times 10^{-1}$	$1.3 \times 10^6$
New Light chain $\alpha$ phOx2	10	$1.5 \pm 0.6 \times 10^{-8}$	$1.7 \pm 0.4 \times 10^{-2}$	$1.1 \times 10^6$
New Light chain and heavy chains				
$\alpha$ phOx34H	16	$2.6 \pm 0.7 \times 10^{-8}$	$7.3 \pm 0.8 \times 10^{-3}$	$2.8 \times 10^5$
$\alpha$ phOx412D	15	$1.5 \pm 0.4 \times 10^{-8}$	$5.8 \pm 0.6 \times 10^{-3}$	$3.9 \times 10^5$
$\alpha$ phOx48A	22	$1.0 \pm 0.2 \times 10^{-8}$	$2.5 \pm 0.2 \times 10^{-3}$	$2.5 \times 10^5$
$\alpha$ phOx312D	20	$6.0 \pm 1.1 \times 10^{-9}$	$3.5 \pm 0.6 \times 10^{-3}$	$5.8 \times 10^5$
$\alpha$ phOx31E	20	$1.1 \pm 0.4 \times 10^{-9}$	$3.8 \pm 0.5 \times 10^{-3}$	$3.5 \times 10^6$

\*Measured by fluorescence quench titration. †Measured by surface plasmon resonance in BIAcore (Pharmacia).

§Calculated from  $k_{on}/K_d$ .

Before selection 0/94 clones bound to phOx whereas after 3 and 4 rounds of selection, 38/94 and 51/94 clones bound to phOx. Supernatants from all 90 clones were screened by SPR for dissociation from phOx-BSA. All 90 clones had slower off-rates than  $\alpha$ phOxB2. These clones were grouped according to off-rate and BstNI restriction pattern and eight clones were sequenced (Fig. 1) revealing 5 unique sequences. All 5 were derived from the same germline VH-gene (VH380.6, ref. 1) as  $\alpha$ phOx-15 and  $\alpha$ phOxB2 but had an additional 5 to 12 residue changes (Fig. 1 and Table 1). Residue 35 was changed from serine to threonine in all 5 mutants.

The affinities of three of the mutants were shown by fluorescence quench titration to be greater than  $\alpha$ phOxB2 (Table 1). The affinities ranged from  $2.6 \times 10^{-8}$  M to  $1.1 \times 10^{-9}$  M (12 to 320-fold higher than  $\alpha$ phOx-15 and 0.6 to 15-fold higher than  $\alpha$ phOxB2). All five mutants had slower off-rates than  $\alpha$ phOx-15 or  $\alpha$ phOxB2. The highest affinity antibody,  $\alpha$ phOx31E, had a faster on-rate than  $\alpha$ phOx-15 or  $\alpha$ phOxB2.

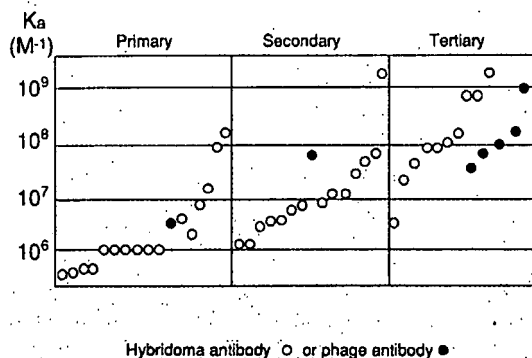
## DISCUSSION

Previously, we used phage display and the V-genes from unimmunized donors to make antibody fragments against both small (hapten) and large (protein) antigens with affinities typical of the primary immune response. While the approach is potentially useful for making therapeutic human antibodies, we need to find ways of increasing the antibody affinities. Here we have shown that this can be accomplished by chain shuffling. We diversified the structure of an antibody by first shuffling light chains, then heavy chains, while retaining the third hypervariable loop of the heavy chain. Much of the sequence and structural variation of antigen binding sites is encoded by this loop, which is located at the center of the antigen binding site<sup>24</sup>. By retaining it, while shuffling the other loops, we aimed to diversify the structure without disrupting the key features of the antigen binding site.

We chose the hapten phOx for our model experiments, as the immune response and affinity and kinetic maturation is well studied<sup>25-28</sup>. The affinities of  $\alpha$ phOxB2, from the light chain shuffled library, and the 5 mutants from the heavy chain shuffled library are comparable to that of mouse hybridomas from the secondary or tertiary immune response to the same hapten<sup>26</sup> (Fig. 2). Indeed, of anti-phOx hybridomas from the mouse secondary or tertiary response, only 2 of 24 had a higher affinity than  $\alpha$ phOx31E (ref. 26).

The improvement in affinity results almost exclusively from a slower off-rate. Somatic hypermutation of the V-genes used in the murine primary immune response to phOx also improves affinity mainly by slowing the off-rate<sup>26</sup>. The results suggest that our washing and binding conditions favor the selection of phages with slower off-rates rather than faster on-rates in contrast to the suggestion of Garrard et al.<sup>29</sup>. As we build antibodies with higher and higher affinities, it becomes increasingly likely that the best binders will remain attached to the solid phase, necessitating more vigorous elution conditions.

*In vivo*, affinity maturation occurs by random mutation of the original heavy and light chain pairings and by the appearance of new heavy and/or light chain pairings (repertoire shift)<sup>27,28</sup>. We can simultaneously mimic aspects of both processes *in vitro* by tapping the natural pool of diverse unmutated and mutated heavy and light chains via chain shuffling. Using V-genes derived from an immunized mouse, we had previously shown that new partners could arise from different V-gene families<sup>2</sup>. In



**FIGURE 2** Comparison of affinities of anti phOx antibodies from hybridomas and from phage antibodies. Affinity constants ( $K_a$ ) for anti-phOx hybridomas from primary, secondary and tertiary responses from immunized mice (data taken from ref. 26) are compared with data (Table 1) for phage antibodies from naive phage library (primary), light chain shuffled (secondary) and heavy chain shuffled (tertiary) libraries.

the present study, both the light chains and heavy chains are derived from the same germline gene and the antibodies differ only by point mutations. Nevertheless the repertoire of mutants should differ from those generated by random mutation of the  $\alpha$ phOx-15 antibody in two respects. Firstly, the V-genes encoding the shuffled chains have been selected from the mRNA of B-lymphocytes and are more likely to be functional. In contrast, *in vitro* random mutagenesis, for example using an error prone polymerase<sup>30</sup>, is likely to result in many mutants that would compromise chain folding, particularly if multiple mutations were introduced into the same gene. Secondly, with *in vitro* mutagenesis mutations are introduced directly into  $\alpha$ phOx-15 whereas with chain swapping, mutations are introduced into the corresponding germline genes. This could allow any deleterious mutations in  $\alpha$ phOx-15 to be replaced more readily.

A shuffling strategy may be applicable to protein antigens as well as haptens. Although there are a larger number of contacts between protein and antibody, and the chances of disrupting multiple favorable contacts by shuffling is greater, this may be compensated by the loss of multiple unfavorable contacts.

One advantage of building an artificial immune system is that by allowing heavy chains to sample other light chains, and vice-versa we employ a strategy that is not open to the immune system. Thus shuffling enlarges the repertoire size, enhancing the chances of finding higher affinity antibodies<sup>30</sup>, and in principle allowing chains with deleterious mutations<sup>31</sup> to be replaced by others. Shuffling chains and hypervariable loops appears to be a powerful way of diversifying antibody structure, and the pool of rearranged V-genes from unimmunized donors provides a rich source of genetic diversity.

## EXPERIMENTAL PROTOCOL

**Construction of a reshuffled light chain library.** A scFv library was assembled<sup>2</sup> from the VH gene of  $\alpha$ phOx-15 and a VL and V $\kappa$  repertoire<sup>1</sup> using PCR. To avoid contamination with the original light chain, the VH gene of  $\alpha$ phOx-15 was subcloned into the vector pJM-1 (ref. 2), amplified by PCR using primers HuVH1aBACK and HuJH6FOR<sup>1</sup>, purified on a 2% (w/v) agarose gel and isolated from the gel using GeneClean (Bio-101). Reshuffled scFv repertoires were PCR assembled<sup>1</sup> from the phOx-15VH DNA, linker DNA and the same human VL and V $\kappa$  gene repertoires used to construct the primary library from

which  $\alpha$ phOx-15 was isolated<sup>1</sup>. The repertoires were digested with NcoI and NotI, purified on a 1.5% (w/v) agarose gel, electroeluted<sup>32</sup>, precipitated with ethanol and ligated into the vector pHEN-1 (ref. 21) digested with NcoI and NotI. The ligation mix was used to transform electrocompetent<sup>33</sup> *E. coli* TG1 (ref. 34). Cells were grown for 1 hour in 1 ml of SOC<sup>32</sup> and then plated on TYE<sup>35</sup> medium with 100  $\mu$ g/ml ampicillin 1% (w/v) glucose. Colonies were scraped off the plates into 5 ml of 2  $\times$  TY<sup>35</sup> broth containing 100  $\mu$ g/ml ampicillin, 1% (w/v) glucose and 15% glycerol.

**Construction of a reshuffled heavy chain library.** A scFv library was prepared containing the VH CDR3 and V $\lambda$  of  $\alpha$ phOx2 and a repertoire of human VH1 genes. To eliminate potential contamination with the original heavy chain, the human VH1 pseudogene DP-22 (ref. 36) was amplified using PCR from an M13 template using the primers HuVH1BACK-SFI (ref. 1) and HuVH1FR3FOR (5'-GGC CGT G/CIC AGA ICT CAG-3'), digested with NcoI and BglII, gel purified and ligated into the vector pHEN-1-phOx2 digested with NcoI and BglII. The resulting vector, pHEN-1-VVHB2, contained the DP-22 VH1 pseudogene and the VH CDR3 and V $\lambda$  of  $\alpha$ phOx2. To prepare a repertoire of human VH1 genes, human PBL RNA was primed in separate reactions with HuIgG1-4CH1FOR and HuIgMFOR and 1st strand cDNA synthesized<sup>1</sup>. The first strand cDNA was used as a template for PCR amplification as previously described<sup>1</sup> using the primers HuVH1aBACK and HuVH1FR3FOR. Restriction sites were appended to the repertoires by reamplification using the primers HuVH1BACKSFI and HuVH1FR3FOR. The VH1 repertoires were digested with NcoI and BglII, purified on a 1.5% (w/v) agarose gel, electroeluted, precipitated with ethanol and ligated into the vector pHEN-1-VVHB2 digested with NcoI and BglII. The ligation mix was used to transform electrocompetent *E. coli* TG1. Cells were grown for 1 hour in 1 ml of SOC and then plated on TYE medium with 100  $\mu$ g/ml ampicillin and 1% (w/v) glucose. Colonies were scraped off the plates into 5 ml of 2  $\times$  TY broth containing 100  $\mu$ g/ml ampicillin, 1% (w/v) glucose and 15% glycerol.

**Selection of reshuffled libraries.** To rescue phagemid particles, 50 ml of 2  $\times$  TY containing 100  $\mu$ g/ml ampicillin and 1% (w/v) glucose (2  $\times$  TY AMP-GLU) were inoculated with 10<sup>9</sup> bacterial cells from the library glycerol stock, grown with shaking at 37°C to an A<sub>550</sub> of 0.9 and then 5 ml added to 50 ml of 2  $\times$  TY AMP-GLU prewarmed to 37°C. 2  $\times$  10<sup>10</sup> plaque forming units of VCS-M13 (Stratagene) were added and the mixture incubated at 37°C without shaking for 1 hour. The mixture was then added to 500 ml of 2  $\times$  TY broth containing 100  $\mu$ g ampicillin/ml and 25  $\mu$ g kanamycin/ml and grown overnight at 37°C with shaking. Phage particles were purified and concentrated as previously described<sup>1</sup>. Two rounds (reshuffled light chain library) or four rounds (reshuffled heavy chain library) of enrichment for phOx binding phage were performed in phOx-BSA coated immunotubes (Nunc) (10  $\mu$ g/ml of 14ox/BSA for selection of the reshuffled light chain library and 10  $\mu$ g/ml of 1ox/BSA for selection of the reshuffled heavy chain library). After each round of enrichment, *E. coli* TG1 were reinfected with eluted phage and rescued to provide phage for the next round of panning. For soluble scFv expression, eluted phage was used to infect *E. coli* HB2151 (ref. 37).

**Initial characterization of binders with new light chains.** Soluble scFv was induced<sup>38</sup> from 94 colonies from each round of selection and analyzed for binding to phOx by ELISA<sup>1</sup>. Twelve clones with ELISA signals stronger than  $\alpha$ phOx-15 were sequenced<sup>39</sup> revealing 8 unique clones. The relative affinities of these 8 clones were determined by inhibition ELISAs. For inhibition ELISAs<sup>22</sup>, microtiter wells were coated overnight with 100  $\mu$ g/ml phOx-BSA in PBS and blocked for 2 hours at 37°C with 2% milk powder in PBS. Dilutions of scFv previously determined to result in significant reduction of ELISA values after two-fold dilution were mixed with phOx (10<sup>-3</sup>–10<sup>-7</sup> M) in the wells and incubated for 1.5 hours at RT. Bound soluble scFv was detected by ELISA<sup>1</sup>. The concentration of phOx resulting in a 50% reduction in ELISA signal (I<sub>50</sub>) was calculated for each mutant and compared to that obtained for  $\alpha$ phOx15 to determine the relative affinity. Relative affinities, but not the I<sub>50</sub> value (6.0–400  $\mu$ M), correlated with affinities measured by fluorescence quench (Fig. 1 and Table 1). Affinities and off-rates of the clone with the highest relative affinity ( $\alpha$ phOx2) as well as  $\alpha$ phOx-15 were determined as described below.

**Initial characterization of binders with new heavy chains.** Soluble scFv was induced<sup>38</sup> from 94 colonies from each round of selection and analyzed for binding to phOx by ELISA<sup>1</sup>. The

off-rates of soluble scFv from all ninety positive clones from the third and fourth round of selection were determined by BIAcore (see below) and the clones then grouped according to off-rate and BstNI fingerprint<sup>1</sup>. Eight representative clones were sequenced<sup>39</sup> revealing 5 unique clones. Affinities and off-rates of these 5 clones were determined as described below.

**Affinity measurements.** Two liter cultures of *E. coli* HB2151 harboring the appropriate phagemid were induced<sup>38</sup> and the soluble scFv affinity purified<sup>2</sup> from the supernatant using the C-terminal peptide tag<sup>40</sup>. For affinity determinations, fluorescence quench titration with the hapten 4- $\gamma$ -amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA) was performed as described<sup>26</sup>. The affinity of  $\alpha$ phOx-15 was determined<sup>26,11</sup> using a regime of hapten excess as described previously<sup>1</sup>. Data were averaged from 3 runs. For determination of the affinity of  $\alpha$ phOx2 and the 5 mutants from the shuffled heavy chain library, 100 nM scFv (a concentration ten times the preliminary estimate of the dissociation constant) was titrated with hapten and the fluorescence determined 1 min after each addition<sup>26</sup>. Excitation was at 280 nm and emission was monitored at 340 nm. Data were averaged from 3 to 5 runs.  $k_{off}$  was measured by real-time biospecific interaction analysis based on surface plasmon resonance (BIAcore, Pharmacia Biosensor AB)<sup>23,24</sup>. Affinity purified scFv proteins were fractionated on a calibrated FPLC Superdex 75 column (Pharmacia) to eliminate aggregates and the monomeric fraction then used for kinetic measurements. In a BIAcore flow cell, 1300 resonance units (RU) of 100  $\mu$ g/ml phOx modified BSA (14 phOx/BSA) in 10 mM acetate buffer pH 4.0 was coupled to a CM5 sensor chip<sup>41</sup>. In another flow cell, the sensor chip was activated without phOx-BSA as a control. Adsorption and dissociation of  $\alpha$ phOx15 (0.4  $\mu$ M–2.8  $\mu$ M) and the other scFvs (80 nM–400 nM) in PBS, 0.2 mM EDTA were measured under a constant flow of 6  $\mu$ l/min.  $k_{off}$  was determined for  $\alpha$ phOx2 and the heavy chain shuffled mutants from the dissociation part of the sensorgram and for  $\alpha$ phOx15 from the association part of the sensorgram<sup>42</sup> (necessitated by its rapid  $k_{off}$ ).

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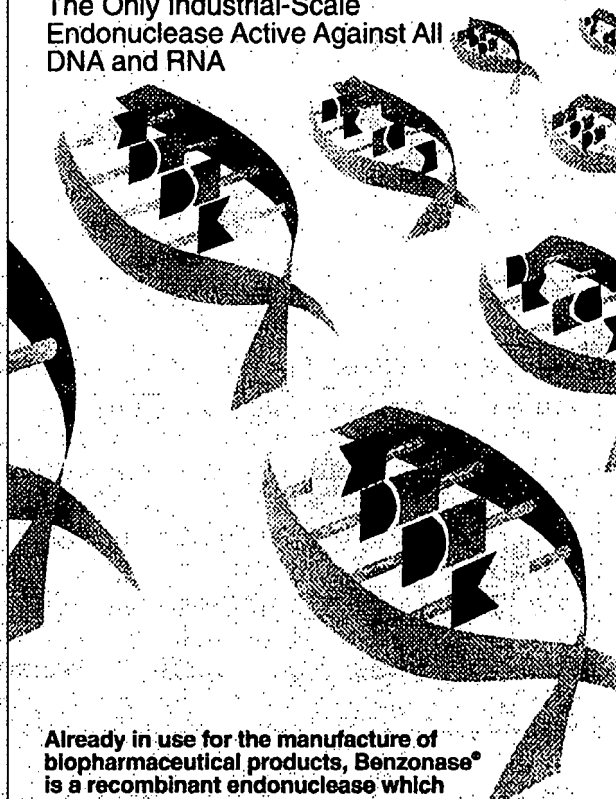
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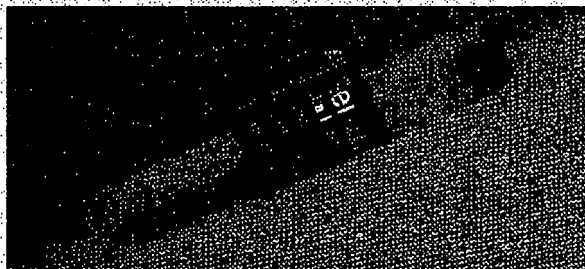
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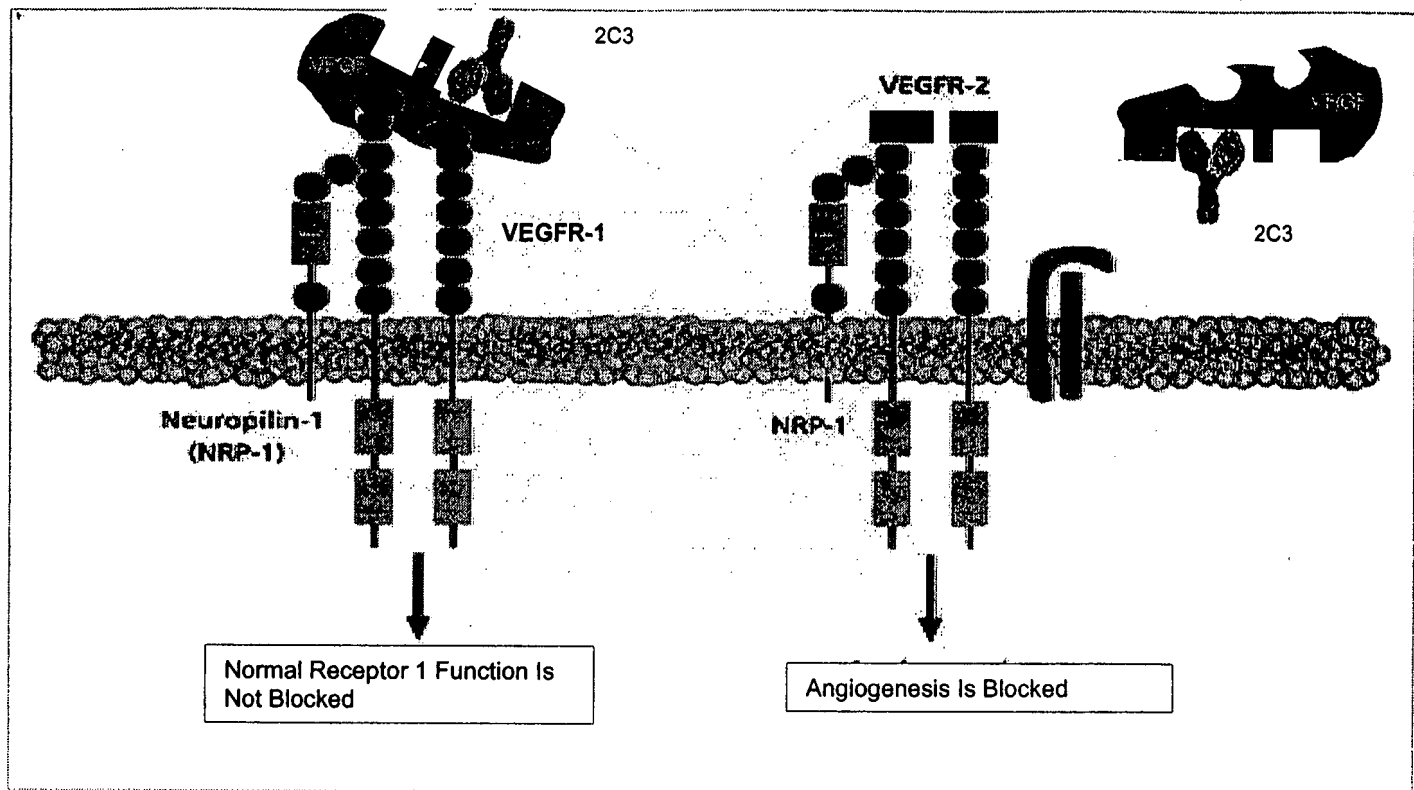
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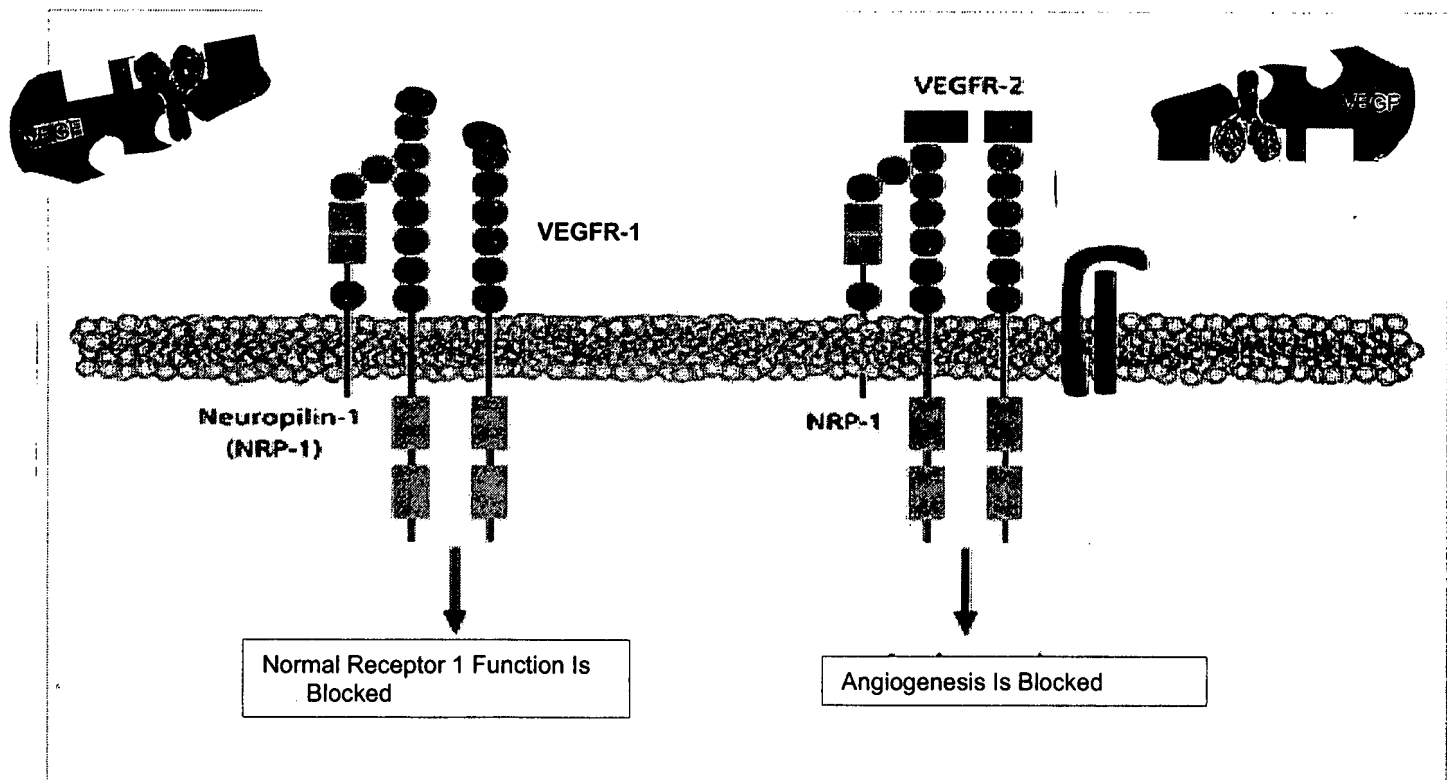
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# EXHIBIT C



**2C3 - VEGF is bound to VEGFR-1 and can be Targeted**



**A4.6.1 - VEGF is not bound to either VEGFR and cannot be Targeted**  
**Humanized A4.6.1**